Proteomic response analysis of endothelial cells of human coronary artery to stimulation with carbachol[1]

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ABSTRACT

AIM: To identify the molecular basis of the endothelial target for acetylcholine (ETA). **METHODS**: Proteomic methods were used to monitor changes in protein expression in the first 10 h following the stimulation of human coronary endothelial cells with carbachol 100 μmol/L. Thirty proteins showing the largest changes were identified by mass spectrometry. **RESULTS**: Based on analysis with Image Master 2-D Elite software, about 623 protein spots were detected in control cells and 825 protein spots in carbachol-treated cells, the matching rate was 68.1 %. Among all the detected spots, 39 were up-regulated and 29 were down-regulated, showing detectable changes varied from 1.7-3.8 folds. Forty one spots in the peptide mass fingerprints were successfully obtained. The most interesting feature was that all the four newly synthesized proteins belonged to the heat shock protein family. **CONCLUSION**: These identified proteins played key roles in the molecular mechanism of ETA.

INTRODUCTION

Endothelium was considered not only a passive barrier lining the inner side of blood vessels, but also one of the largest secretory tissues of the body[1]. Endothelial cells were responsible for the regulation of a variety of biological functions such as the secretion and release of various active substances and the control of the vascular tone[1,2]. Studies indicated that the endothelium dysfunction might play key roles in many cardiovascular diseases, such as atherosclerosis, hypertension, and hyperlipidemia[3]. Therefore, endothelial cell was an essential target for the etiological factor of various cardiovascular diseases; on the other hand, it was also the elemental field for the development of new drugs for both the prevention and therapy of corresponding illnesses. Endothelial target for acetylcholine (ETA) was widely expressed in vascular endothelium, and the endothelium-dependent relaxation mediated by ETA was considered to be the classical indicator of the endothelium dysfunction[4]. In those vascular diseases we referred to, the endothelium-dependent relaxation in response to acetylcholine appeared to be impaired, ie, ETA function was down-regulated. According to these, ETA might be probably developed into a novel drug target contributed to those diseases. Our previous study suggested that different from classical muscarinic receptors, ETA displayed their own distinct pharmacologic characteristics[5]. To date, however, the signal

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transduction mechanism was still obscure after the activation of ETA, and the molecular biology nature of ETA was not addressed.

In this present study, with the use of the high sensitivity of both two-dimensional electrophoresis and mass spectrometry, which was comprehended in the emerging field of proteomics[6,7], we analyzed and identified the proteins with changed expression upon the stimulation with carbachol in the cultured human coronary artery endothelial cells (HCAEC). This proteomic approach might contribute to an understanding of the signaling events relevant to ETA.

MATERIALS AND METHODS

Materials Human coronary artery endothelial cell lines and endothelial cell medium-2 [EGM-2, containing fetal bovine serum (FBS) and seven kinds of growth factors] were from American BioWhittaker Company. Trypsin, carbachol, and Coomassie Brilliant Blue R-250 were from Sigma. Rabbit anti-Factor VIII related antigen polyclonal antibody kit was from Denmark DAKO. Acrylamide (40 %), dimethylformamide, TEMED, ammonium persulphate, SDS, Tris, dithiothreitol, glycine, CHAPS, agarose, molecular weight marker, IPG buffer, Immobiline™ DryStrips (pH 3-10), and Immobiline™ DryStrip cover fluid were from Amersham Pharmacia Biotech. Iodoacetamide were from Acros, Japan.

Cell culture and identification HCAEC cells (4th passage)were cultured in EGM-2 supplemented with 5 % FBS and seven growth factors in a water-saturated atmosphere with 5 % CO_2 and at 37 ºC in 75-cm^2 polystyrene culture flask. Nearly confluent HCAEC cells were digested with 0.1 % trypsin for passage. The cells were transferred to the eighth passage when the total number was enough (2×10^7) to perform the experiment. The purity of the endothelial cells was identified by the monolayers of cobblestone morphology and staining of Factor VIII as a cellular marker.

Carbachol induction and sample preparation The cells were incubated with or without carbachol (control) 100 μmol/L at 37 ºC for 10 h. After induction, the cells were digested with trypsin and washed with D-Hanks' solusion for three times. After centrifugation at 4000 rpm at 4 ºC for 2 min, the cells were resuspended in CHAPS extraction buffer containing protease inhibitors 2 μL, DTT 4 μL, pharmalyte 2 μL, Tris base buffer 40 mmol/L, urea 8 mol/L, and 4 % CHAPS. After being lysed by frozen and melt in liquid nitrogen three times, the samples were added 4 μL RNA protease inhibitor and icebathed for 20 min. The supernatant was stored at -80 ºC followed by centrifugation at 12 000×g at 4 ºC for 30 min. The protein content of the sample was calculated by Coomassie Brilliant Blue R-250 assay.

2-D electrophoresis Essentially according to Gorg[8], and following the manufacture's instruction, two-dimensional electrophoresis was performed. For the first dimension, the solubilized sample was mixed with a rehydration solution containing urea 8 mol/L, 2 % CHAPS, 0.5 % immobilized-immobilized-pH-gradient (IPG) buffer (pH 3-10), DTT 60 mmol/L, and a tracer of bromophenol blue, to a total volume of 300 μL, and subjected to isoelectic focusing (IEF) on an IPG strip (pH 3-10, 18 cm). The strips were run under a layer of Drystrip cover fluid and loaded onto the IPGphor. After 12-h rehydration, IEF were conducted automatically on the IPGphorplatform at 20 ºC, with IEF up to a total of 70 kVh. Prior to SDS gel electrophoresis, the strips were equilibrated for 15 min in an equilibration buffer [Tris-HCl 50 mmol/L (pH 8.8), urea 6 mol/L, 30 % glycerol, 2 % SDS, and a tracer of bromophenol blue) containing DTT 20 mmol/L and subsequently in a solution containing iodoacetamide 100 mmol/L in the same buffer for 15 min. For the second dimension, the strips were placed onto the top of the vertical 12 % SDS polyacrylamide gels and sealed with 0.5 % agarose. SDS-PAGE was carried out at a constant current of 20 mA at 14-15 ºC. Then the constant current was switched to 30 mA until the front of bromophenol blue reached the bottom of the gel. Low molecular weight protein was added as marker to identify the protein spot's molecular weight.

2-D protein gel staining Silver staining and Coomassie Brilliant Blue staining were performed according to the methods described in the reference[9,10].

Image acquisition and analysis Gels were scanned with a computer-assisted densitometry. Qualitative analysis of digitized images was performed with Image Master 2-D Elite software. Image analysis included the following procedures: spot detection, spot editing, background subtraction, and spot matching. The gels in the treatment group were chosen as a reference gel before matching. The amount of a protein spot was expressed as the volume of the spot, which was defined as the sum of the intensities of all the pixels that made up of the spot.

In gel digestion and mass spectrometric analysis Difference in expression was confirmed by visual inspection prior to spot picking for mass spectrometry. Selected protein spots were excised from the gel and in-gel digestion with trypsin was performed according to the procedures described in the reference[11] with slight modification. Spectra were obtained using a Bruker Biflex MALDL-TOF mass spectrometer. For identifying the protein we used the MASCOT program available at the Matrix Science web site (http://www. Matrix science.Com), the Profound program at the web site of Rockefeller University (http: //prowl.Rockefeller. Edu/cgi-bin/Profound). Searches were carried out with the following parameters: at least mass of four peptides was required to match; MS/MS ion mass tolerance, 0.5 Da; allow up to one missed cleavage; variable modifications considered were methionine oxidation and cysteine carboxyamidomethylation.

RESULTS

The identification of HCAECs Under an inverted microscope, the HCAECs showed a typical polygonal cobblestone-like shape (Fig 1A); the immunohistochemistry revealed the positive response of the factor VIII (Fig 1B).

2-D protein patterns of HCAECs A pair of silver-stained gels obtained from the control and carbachol-induced group separately was displayed (Fig 2, 3). Fig 4 showed a representative of the comparison of protein spots between the control and carbachol treatment group. Altogether three pairs of gels from the same two group proteins samples were analyzed which were conducted in the same electrophoresis condition. Of these three sets of gels, one was viewed with silver staining using 100 μg of total proteins, while two others were viewed with Coomassie Blue staining using 500 μg and 900 μg of total proteins separately, all of which showed similar protein patterns. Based on analysis with Image Master 2-D Elite software, about 623 protein spots were detected in control cells and 825 protein spots in carbachol-treated cells, the matching rate was 68.1 %. Among all the detected spots, 39 were up-regulated and 29 were down-regulated, showing detectable changes varied from 1.7-3.8 folds. Also newly synthesized protein spots were observed.

Proteins with altered expression profiles identified by MALDI-TOF and database searching Keeping the silver-staining gels as a reference, we selected 41 well-stained, clearly-delineated, and marked spots

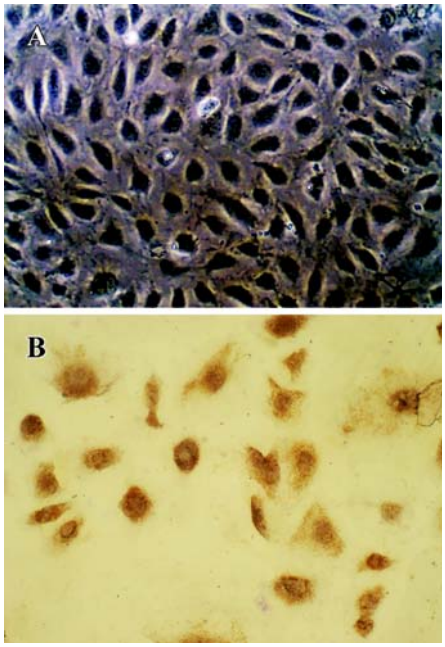

Fig 1. Characterization and identification of the cultured human coronary artery endothelial cells (HCAECs). (A) In 1 week, cells form the regular monolayer of cobble stone shape. (B) HCAECs are characterized by immunohistochemistry with the use of anti-factor VIII antibody. A and B are all observed with the inverted microscope ×100 magnification.

that showed remarkable changes. In all the selected spots 29 were up-regulated spots, 8 were down-regulated spots, and 4 were newly-synthesized spots. These spots were excised from the corresponding gels and digested with trypsin and the digests were analyzed by MALDI-TOF. The peptide mass fingerprints (PMF) of all the 41 spots were successfully obtained. A selected MALDI mass spectrum for the tryptic digests of spot 49 was presented in Fig 5. Database searches with Mascot and Profound were performed using recorded peptide mass fingerprint data sets (excluding known keratin masses and trypsin autodigest products). The result had high confidence if the protein was ranked as the best hit with a significant score and high sequence coverage. We identified 30 of these 41 proteins. The remaining 11 protein spots were not successfully identified by the PMF, which was probably due to the fairly perfect quality of the database and the poor nature of their PMF. The identified proteins were listed in Tab 1.

DISCUSSION

This study tried to explore the signaling mechanism and related proteins of ETA directly at the protein

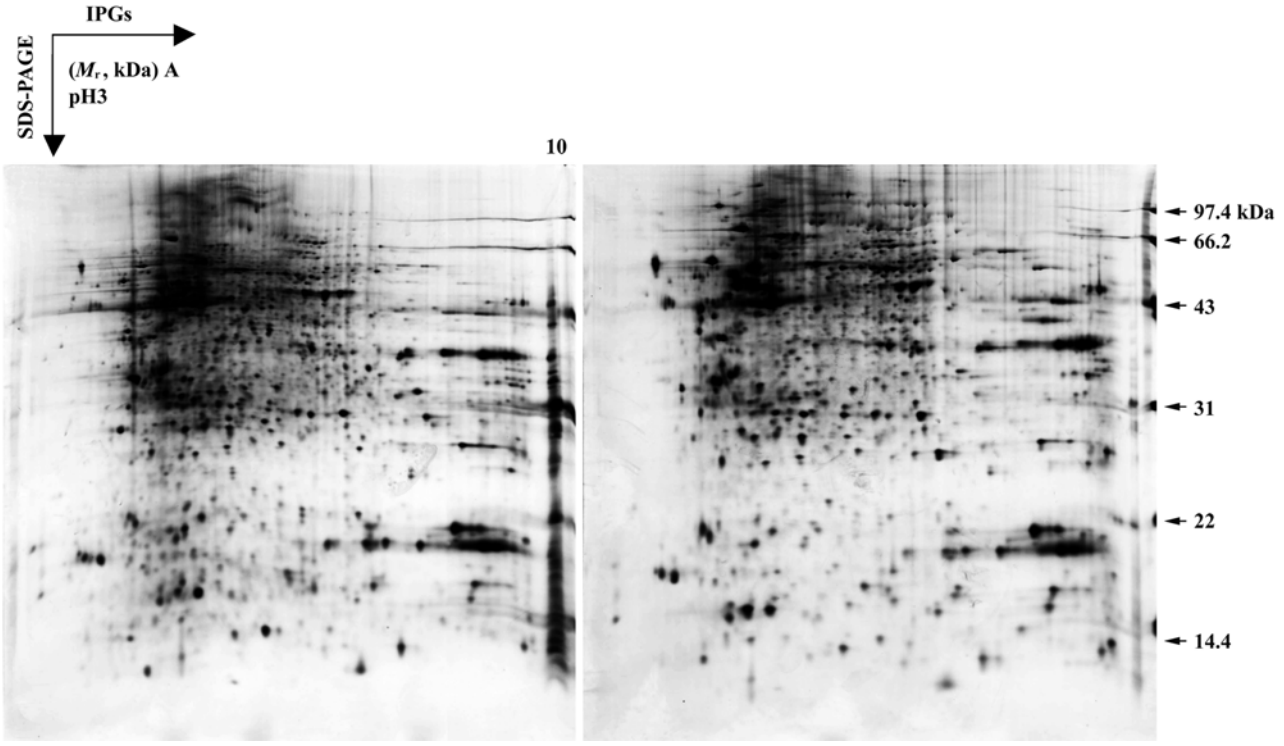

Fig 2. Two-dimensional electrophotograms of total proteins (100 μg) from human coronary cells following stimulation without (A) and with carbachol 100 μmol/L stimulation; (B) for 10 h. The gels were stained with silver.

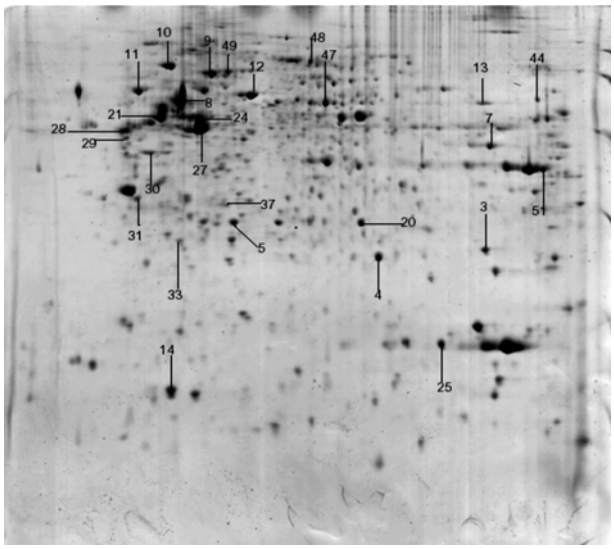

Fig 3. Two-dimensional gel protein expression patterns of cultured HCAECs (500 μg of protein) after stimulation with carbachol 100 μmol/L for 10 h. The protein spots were visualized with Coomassie Brilliant Blue staining. Numbered spots that had been identified were given in Tab 1.

level by comparing the altered protein profiles between the control and treatment group, using the stable analog of acetylcholine, carbachol, to activate ETA on HCAECs.

There were several important aspects of our experimental scheme. First, the identity of the experiment was confirmed using the same batch of the cultured 8th HCAECs, which had the capacity to activate ETA successfully. Second, the selected concentration and the induction time were decided mainly according to our previous study and the references, under which condition ETA could still be successfully activated while abundance of non-specific substances that downstream the signaling way of ETA could be avoided. Third, to identify the membrane protein, it was necessary to enrich its low abundance before 2-D electrophoresis as well as a high sensitivity to detect them. As described in materials and methods, the protein samples were solubilized to large extent concerning the protein preparation procedures. In addition to this, silver-staining gels with high sensitivity were combined with Coomassie Blue staining gels, which had fine reproduction and could be used as quantitative analysis. And finally, the analyzed protein spots were all those displayed the change above 1.7 fold or the newly-synthesized proteins. Therefore, it was reasonable to suggest that those proteins were directly related to the response of ETA.

We observed that activation of ETA changed the expression levels of different groups of cellular proteins including cytoskeleton and associated proteins

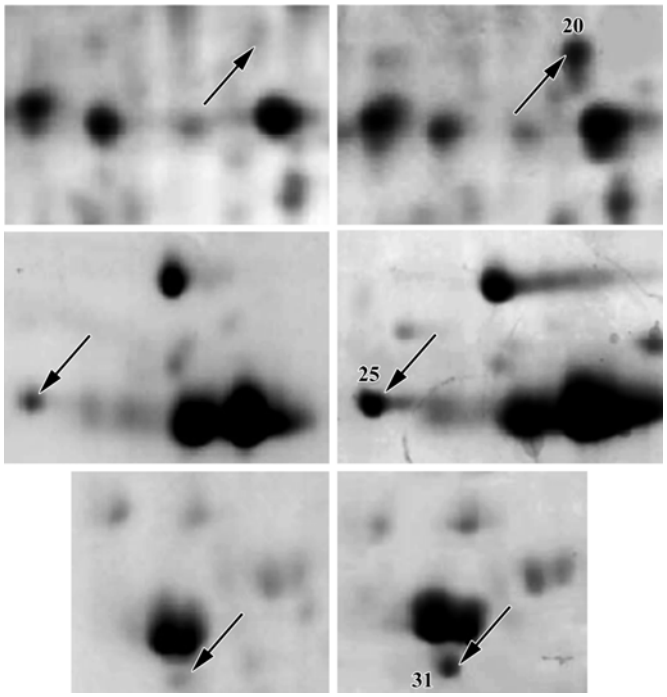

Fig 4. Insets from Fig 3 represented changed patterns between HCAECs treated with and without carbachol 100 μmol/L.

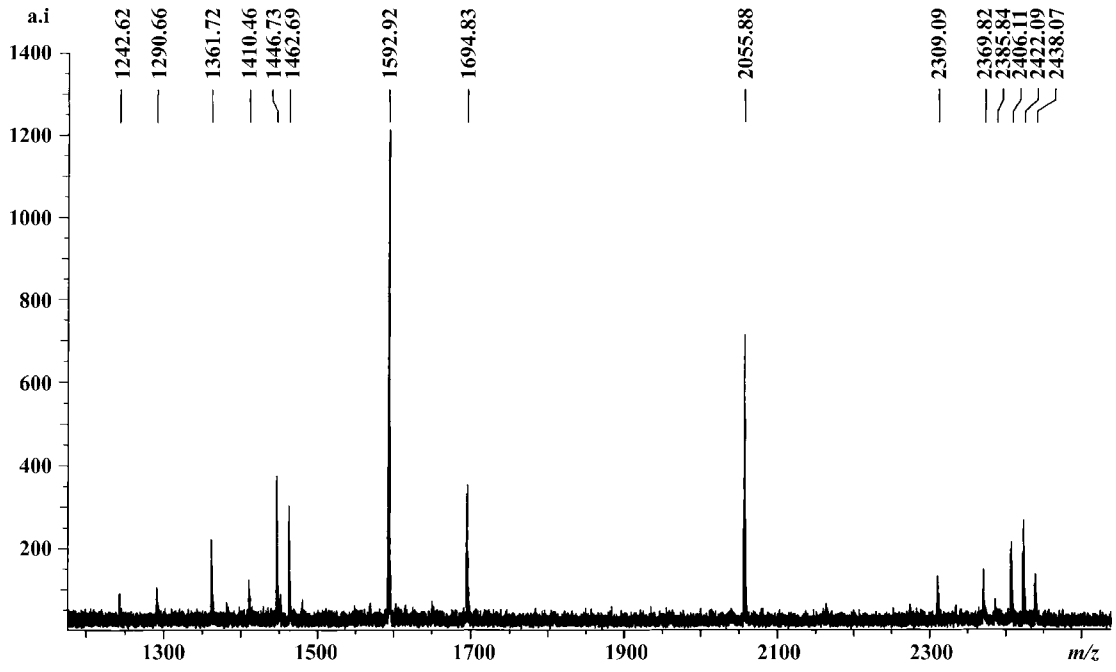

Fig 5. Peptide mass finger print of protein spot 49 (heat shock protein gp96 precursor) obtained from MALDI-TOF-MS.

(vimentin and actin); signaling-associated proteins (reticulocalbin 1 precursor, and caldesmon); proteins involved in anti-oxidation and proliferation (proliferation-associated gene A, prohibitin, signal-regulatory protein beta 1 precursor, thioredoxin-related protein, peptidyl-prolyl isomerase A, and beta-galactosidase binding lectin precursor); as well as glycolytic proteins (Aldolase A, Alpha enolase, and aldehyde dehydrogenase 1A1). Five spots were identified as unknown proteins.

However, a group of heat shock proteins (heat shock 70 kDa protein 8 isoform 1, heat shock 70 kDa protein 5, heat shock protein gp96 precursor, and heat

Tab 1. Proteins identified by mass spectrometry.

Spot No	Identified protein	NCBI entry	M_r kDa/pI Experimental	Predicted	Peptides*	% Sequence	Mascot	Pro-found
9*	Heat shock 70 kDa protein 8 isoform 1	5729877	69.82/5.40	70.85/5.37	10/18	27	++	
10*	Heat shock 70 kDa protein 5	16507237	71.21/4.89	72.29/5.07	10/15	23	+	+
20*	Heat shock protein gp96 precursor	15010550	75.35/4.66	90.14/4.73	4/7	9	++	
49*	Heat shock 70kDa protein 9B precursor	24234688	73.07/5.68	73.64/5.87	9/15	18	++	
3↑	Proliferation-associated gene A	4505591	37.26/8.68	22.10/8.27	8/14	52	++	
7↑	Fructose-bisphosphate aldolase A	4557305	56.50/8.74	39.40/8.30	4/14	27	–+	
8↑	Vimentin	2119204	65.23/5.04	53.62/5.06	11/28	33	+	+
11↑	protein disulfide-isomerase¡¡precursor	35655	66.577/4.53	57.06/4.77	8/14	25	++	
12↑	Protein disulfide-isomerase ER60 precursor	2245365	65.86/5.89	56.75/5.88	8/18	23	++	
13↑	Unknown protein	14125001	64.37/8.67	49.38/9.17	6/11	19	+	+
14↑	Beta-galactosidase binding lectin precursor	4504981	11.31/4.92	14.71/5.34	4/15	42	++	
21↑	Vimentin	211904	63.95/4.91	53.62/5.06	18/40	49	+	+
24↑	Thioredoxin domain containing 5	13540604	62.88/5.34	36.16/5.32	10/26	31	++	
25↑	Peptidylprolyl isomerase A (cyclophilin A)	10863927	14.25/8.18	18.00/7.68	8/29	65	++	
27↑	Unknown (protein for MGC: 9832)	15277503	59.74/5.37	40.19/5.55	10/27	46	++	
28↑	Reticulocalbin 1 precursor	4506455	59.46/4.40	38.87/4.86	8/26	38	+	+
29↑	Reticulocalbin 1 precursor	4506455	57.56/4.39	38.87/4.86	10/24	42	+	+
30↑	Signal-regulatory protein beta 1 precursor	5174679	53.28/4.75	43.23/6.18	4/14	23	++	
31↑	Unnamed protein product	16552261	42.95/4.65	47.46/5.01	6/14	24	+	
33↑	Histamine N-methyltransferase	7512484	36.83/5.19	33.27/5.18	3/10	23	++	
34↑	Unknown protein	15928913	38.60/5.46	21.23/5.55	5/15	29	+	+
37↑	Prohibitin	4505773	41.78/5.74	29.79/5.57	13/26	58	+	+
44↑	Peroxisomal trans 2-enoyl CoA reductase	19923817	66.23/9.42	32.52/8.97	3/7	18	++	
47↑	Aldehyde dehydrogenase 1A1	21361176	67.37/6.79	54.83/6.30	12/22	36	+	+
48↑	Caldesmon	179830	76.08/6.6	62.66/6.18	4/12	15	+	–
51↑	Heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	4504447	45.54/8.61	35.98/8.67	9/19	48	+	+
64↓	Unnamed protein product	16552261	61.67/4.91	61.67/4.91	47.46/5.01	11/19	38	++
15↓	Alpha enolase	4503571	61.61/6.69	47.14/7.01	5/17	21	+	+
22↓	Peptidylprolyl isomerase A	10863927	14.42/7.42	18.00/7.68	8/24	66	+	+

Peptides*: Number of matched peptides/Number of the total queries.
↑ The protein up-regulated after stimulation of the ETA.
↓ The protein down-regulated after stimulation of the ETA.
* The protein newly synthesized after stimulation of the ETA.
+ Successful identification of the protein using peptide mass fingerprint data.
– Unsuccessful identification of the protein using peptide mass fingerprint data.

shock 70 kDa protein 9B precursor) was most significantly induced by carbachol. Heat shock proteins 70 (HSP70) were non-specific cyto-protective proteins. Pretreatment of cells with a mild stress, sufficient to induce the expression of HSP70, resulted in protection against subsequent insults[12]. The identified HSP70 in endothelium in this study were thought to be related to some second messengers including free calcium and protein kinases. The induction of heat shock protein 5, also termed as glucose-regulated protein 78 (GRP-78) was reported to be associated with a protein kinase C-epsilon/ERK/AP-1 signaling cascade after chronic hypoxia in human gastric tumor cells[13]. These suggested that the new synthesis of HSP was likely to be well connected with the effective enzyme of ETA. Also calcium-binding proteins in these up-regulated proteins suggested that calcium ions were probably one of the signaling substances involved in the signaling way of

ETA.

In addition, another group of the identified up-regulated proteins which included a large number of proteins were involved in antioxidation and cell proliferation. The negative roles of peptidylprolyl isomerase A [14] and signal-regulatory protein beta 1 precursor[15] on cell proliferation were through tyrosine kinase signaling pathways. It might be helpful to explain the role of ETA in retarding the atherosclerosis progress.

We used the 2-D proteomic approach with high sensitive mass spectrometry to analyze changes in protein expression of HCAECs induced by carbachol treatment. Although it was not safe to address that we had characterized all the protein changes after stimulation of ETA and it was not possible to comment here all the detected proteins had remarkable changes, the above examples were sufficient enough to manifest that our experiments revealed some proteins that were directly associated with the function and substantial signal way of ETA. There were several unknown proteins that need further study after being sequenced. Because of limitation of the 2-D proteomic approach to detect the low-abundance of transmembrane proteins, it was harder to compare this technique with the nowadays-genomic ones. Further study in other ways should be performed to facilitate the understanding of ETA.

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